



Synthetic addiction extends the productive life time of engineered *Escherichia coli* populations

Rugbjerg, Peter; Sarup-Lytzen, Kira; Nagy, Mariann; Sommer, Morten Otto Alexander

Published in:

Proceedings of the National Academy of Sciences of the United States of America

Link to article, DOI:

[10.1073/pnas.1718622115](https://doi.org/10.1073/pnas.1718622115)

Publication date:

2018

Document Version

Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

Citation (APA):

Rugbjerg, P., Sarup-Lytzen, K., Nagy, M., & Sommer, M. O. A. (2018). Synthetic addiction extends the productive life time of engineered *Escherichia coli* populations. *Proceedings of the National Academy of Sciences of the United States of America*, [29463739]. <https://doi.org/10.1073/pnas.1718622115>

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Synthetic addiction extends the productive life time of engineered *Escherichia coli* populations

Peter Rugbjerg^a, Kira Sarup-Lytzen^a, Mariann Nagy^a, and Morten Otto Alexander Sommer^{a,1}

^aNovo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, DK-2800 Kongens Lyngby, Denmark

Edited by James J. Bull, The University of Texas, Austin, TX, and approved January 29, 2018 (received for review October 25, 2017)

Bio-production of chemicals is an important driver of the societal transition toward sustainability. However, fermentations with heavily engineered production organisms can be challenging to scale to industrial volumes. Such fermentations are subject to evolutionary pressures that select for a wide range of genetic variants that disrupt the biosynthetic capacity of the engineered organism. Synthetic product addiction that couples high-yield production of a desired metabolite to expression of nonconditionally essential genes could offer a solution to this problem by selectively favoring cells with biosynthetic capacity in the population without constraining the medium. We constructed such synthetic product addiction by controlling the expression of two nonconditionally essential genes with a mevalonic acid biosensor. The product-addicted production organism retained high-yield mevalonic acid production through 95 generations of cultivation, corresponding to the number of cell generations required for >200-m³ industrial-scale production, at which time the nonaddicted strain completely abolished production. Using deep DNA sequencing, we find that the product-addicted populations do not accumulate genetic variants that compromise biosynthetic capacity, highlighting how synthetic networks can be designed to control genetic population heterogeneity. Such synthetic redesign of evolutionary forces with endogenous processes may be a promising concept for realizing complex cellular designs required for sustainable bio-manufacturing.

genetic heterogeneity | population dynamics | synthetic biology | scale-up

Engineering of microbial cells to produce chemicals cost-effectively is emerging as a promising alternative to petrochemical synthesis and natural extraction. However, heavily engineered high-performing strains remain difficult to scale for robust long-term production at industrial fermentation volumes (e.g., 1- to 200-m³ bioreactors) (1, 2). Reduced bioreactor yields are commonly ascribed to spontaneous establishment of nongenetic single-cell variation, in which subpopulations intermittently limit production due to phenotypic noise such as stochastic gene expression and physical mass transfer heterogeneities (3–5). However, engineered strains frequently also exhibit reduced cell fitness due to the loads of production. Production loads typically arise from metabolic depletions (6) in addition to toxicities from pathway intermediates and end products (7). Such production loads can cause declining yields due to genetic instabilities and eventually lead to the complete loss of production, posing substantial challenges to the scale-up of bio-based processes (8–10) and applicability of continuous fermentations (11, 12). Optimization of long-term genetic strain stability is therefore a necessary and research-intensive process toward commercialization of bioprocesses.

One strategy to improve production and also limit the production load by reduction of flux imbalances and pathway intermediate toxicities is to deploy pathway promoters that respond to product stress or precursor supply (13–15). Adaptive laboratory evolution represents an alternative strategy, which has been successfully used to reduce the fitness defects of engineered strains (16). Still, production loads are challenging to eliminate.

Metabolite-responsive molecular biosensors have been developed to accelerate the screening process for new or optimized enzymatic activities and to interrogate metagenomics libraries (17–19).

Biosensor-based screening of libraries >10⁸ is possible by deploying metabolite-responsive screening circuits with fluorescence or selection outputs at low false-positive levels (20). By employing prototrophic or antibiotic selection genes, a biosensor has also been used in a production strain to enrich a phenotypically high-performing subpopulation (5). However, such medium-conditional selection genes are at risk for cross-cell leakage of the conditional nutrients or antibiotic-degrading enzymes and constrain the medium composition in ways that can be prohibitive for large-scale fermentations.

As an approach to synthetically limit the abundance of non-producing mutant cells in a population, we propose a synthetic product addiction system that punishes nonproducing mutants. Unlike screens, a population control system should function without conditioned growth medium, since such conditions are poorly compatible with large-scale industrial fermentations (1). Instead, we propose that the system could harness the regulation of essential endogenous cell processes to punish nonproducing cells in the population. In this study, we develop such a synthetic product addiction system for a high-yielding mevalonic-acid-production organism and experimentally test the system under long-term cultivation conditions designed to mimic the exponential growth durations of large batch or continuous industrial-scale bioreactors.

Significance

Bioproduction of chemicals offers a sustainable alternative to petrochemical synthesis routes by using genetically engineered microorganisms to convert waste and simple substrates into higher-value products. However, efficient high-yield production commonly introduces a metabolic burden that selects for subpopulations of nonproducing cells in large fermentations. To postpone such detrimental evolution, we have synthetically addicted production cells to production by carefully linking signals of product presence to expression of nonconditionally essential genes. We addict *Escherichia coli* cells to their engineered biosynthesis of mevalonic acid by fine-tuned control of essential genes using a product-responsive transcription factor. Over the course of a long-term fermentation equivalent to industrial 200-m³ bioreactors such addicted cells remained productive, unlike the control, in which evolution fully terminated production.

Author contributions: P.R. and M.O.A.S. designed research; P.R., K.S.-L., and M.N. performed research; P.R., K.S.-L., and M.O.A.S. analyzed data; and P.R., K.S.-L., and M.O.A.S. wrote the paper.

Conflict of interest statement: P.R., K.S.-L., and M.O.A.S. are inventors on a patent application (EP15187150) covering the concept of product addiction. The research leading to these results has received funding from the Novo Nordisk Foundation, Denmark, and from the European Union Seventh Framework Programme (FP7-KBBE-2013-7-single-stage) under Grant Agreement 613745, Promys.

This article is a PNAS Direct Submission.

This open access article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

Data deposition: Deep-sequencing data from the study have been deposited in ArrayExpress (accession no. E-MTAB-6182).

¹To whom correspondence should be addressed. Email: msom@bio.dtu.dk.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1718622115/-DCSupplemental.

Results

To engineer durable and effective product addition (Fig. 1A), we define three design criteria that address general large-scale bioprocess constraints. Design criterion 1: Cells should be non-conditionally addicted to product, i.e., excluding use of antibiotic resistance and prototrophic selection genes. Large-scale bioreactors operate with strict economic and regulatory requirements prohibitive of inducers or specific nutrient omissions that are normally attractive in laboratory-scale molecular engineering. Accordingly, common inducers such as isopropyl β -D-1-thiogalactopyranoside (1), drop-outs of nutrients, or addition of antibiotics are often not economically viable to implement in large-scale bioprocesses. Design criterion 2: Product addition should not reduce biosynthetic yield and titer. Pleiotropic effects of product addition might occur as consequence of perturbing expression of genes involved in central transcription, translation, and glucose metabolism limiting the commercial viability of the addition system. Design criterion 3: Product addition should pose no or limited basal fitness cost. A system designed for controlling microbial growth potentially reduces cellular fitness even at the permissive ideal conditions of active production by resource depletion or stresses (21, 22) or suboptimal expression tuning. Such fitness costs might lower biosynthetic performance and could positively select for mutations that inactivate the addition system, which in turn would permit subsequent production declines.

To render the growth control system unconditional (design criterion 1), we targeted genes encoding cellular enzymes outside of the primary metabolism and chose the chromosomal gene operon of *glmM* and *folP* (Fig. 1B) after testing the direct addition ability of four different essential operons or single genes (SI Appendix, SI Text 1). Not previously applied for selections, both phosphoglucosamine mutase (GlmM) and dihydropteroate synthase (FolP) are necessary for cell growth in complex and minimal medium. GlmM supplies essential glucosamine-1-phosphate

for biosynthesis of lipopolysaccharide and peptidoglycan of the cell envelope (23). FolP is involved in tetrahydrofolate biosynthesis (24), and thus is also a possible unconditional growth regulator.

Dose-Sensitive Metabolite Addition with Minimal Burden. We first tested the dose-sensitivity of the *folP-glmM* operon for regulating growth when transcriptionally controlled by the AraC transcription factor in response to its well-characterized inducer L-arabinose (25). In our designs, we assumed that careful molecular titration of the actuating essential gene is critical for efficient control of growth: too high basal expression may result in limited or no control of growth, whereas contrasting low basal expression causes growth reductions (fitness cost) even in the presence of intentional intracellular product concentrations. Therefore, to limit the basal burden of the system (design criterion 3) and also balance expression to a level of dose-sensitive growth regulation, we generated a small pool of translational and transcriptional variants in the ribosome-binding site (RBS) strengths for *folP* and the product-responsive promoter (Fig. 2A). We thus generated clone candidates by swapping the native essential gene operon promoter with two responsive promoter (pBAD) variants using lambda Red recombineering (26) (Materials and Methods). Within the integration DNA constructs, we simultaneously introduced four different RBSs (SI Appendix, Table S4) for *folP*, selected to span an interval in the low-expression region (Fig. 2A) (27) to further increase chances of selecting an ideally tuned clone. Due to our intended, nonconditional product dependency, we directly fed the transformed clone candidates with 0.1–0.5% L-arabinose in LB agar plates and liquid medium (Materials and Methods). Indeed, in minimal M9 medium, we observed different growth rates of the resulting clones, which matched the different RBS strengths (Fig. 2B and SI Appendix, Table S4). This indicated that *folP* was regulating growth at dynamic levels. Three clones with high fitness, *e3.5*, *e3.5m*, and *e3.6*, displayed addition to L-arabinose in liquid cultures (Fig. 2B) with various degrees of system fitness cost, compared with the control strain *AraC* with wild-type *folP-glmM* promoter. Clones with lower predicted *folP* RBS strength were still L-arabinose-responsive yet screened out due to much slower growth and, consequently, higher escape propensity. In the absence of L-arabinose, *e3.5m* displayed >40% reduction in average growth rate (Fig. 2B), indicating significant addition to L-arabinose. Furthermore, *e3.5m* appeared to accommodate our design criterion 3 by very little growth restriction in the presence of the sensed metabolite (Fig. 2B). We further validated the growth response in complex tryptone-based (2xYT) medium to demonstrate that addition was not dependent on nutrients lacking in our M9 minimal medium (SI Appendix, Fig. S1).

Synthetic Addition to Mevalonic Acid Biosynthesis. Utilizing the least fitness-costly design for L-arabinose addition (*e3.5m*), we next wanted to develop a mevalonic-acid-addicted production strain by shifting the biosensor to the mevalonic-acid-responsive *AraC_{mev}* (28). Mevalonic acid is an attractive chemical building block for products such as fragrances and plastics and with available high-performance heterologous pathways in *Escherichia coli* (29, 30). *AraC_{mev}* responds to mevalonic acid starting at 10 mM (1.5 g/L) exogenous mevalonic acid (28). Since intracellular requirements should be lower, we hypothesized that this system could match the productivities of current *E. coli* mevalonic acid pathways at around 0.3 g/L/h (30). Due to our nonconditional mevalonic acid addition design, we wanted to ensure early, sensor-saturating levels of mevalonic acid in the production strain. We therefore engineered a constitutive version of a known mevalonic acid pathway based on overexpressed *E. coli* *atoB* and *Lactobacillus casei* *mvaS* and *mvaE* (30). Next, we also supplied *araC_{mev}* and finally recombineered the pBAD-RBS-*folP-glmM* design of *e3.5m* into the chromosome. The resulting strain, *e3.9*, would readily form colonies in the absence

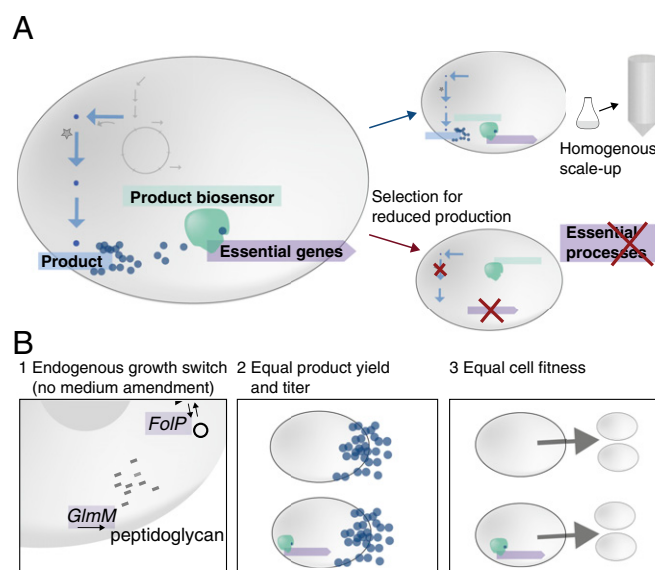


Fig. 1. Concept and design criteria of product addition to prevent evolution of an engineered pathway. (A) In product addition, production cells are equipped with a genetically encoded product biosensor that drives expression of essential cell processes in response to product presence. This limits growth of spontaneously nonproducing mutant cells, preventing evolution. (B) The addition system is designed following three criteria as shown: (design criterion 1) control of growth using nonconditionally essential processes, catalyzed by tetrahydropterate synthase FolP and phosphoglucosamine mutase GlmM in cell-wall peptidoglycan biosynthesis, as these processes function independently of medium additions or omissions and should be tuned to (2) not reduce basal product titer and yield and (3) not reduce growth rate under ideal product presence to avoid escape from the addition.

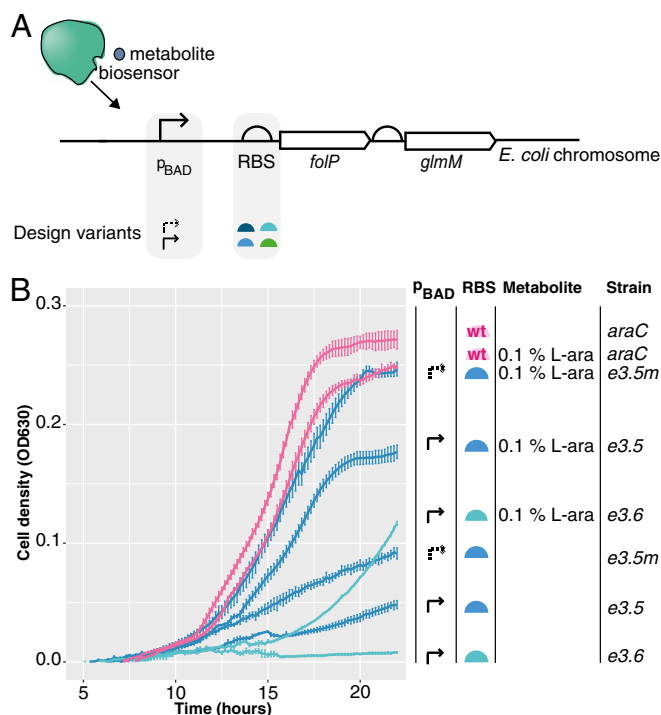


Fig. 2. Engineering of product-addicted cells. (A) Genetic design of the addicted strains using a biosensor and replacing a native essential gene promoter with metabolite-responsive promoter (two variants) and RBS (four variants) strengths for the first essential gene. (B) Identification of responsive, burden-free designs of *E. coli* XL1 *araC* cells with L-arabinose-responsive *p*_{BAD} promoter regulating *folP-glmM* expression at low (*e3.5*) and lower (*e3.6*) predicted *folP* RBS strengths, as well as the stronger promoter variant *p*_{BAD}* (*e3.5m*), shown next to *E. coli* XL1 *araC* for evaluation of basal burden. Growth observed in standard M9 medium in the presence and absence of 0.1% L-arabinose at 30 °C (Materials and Methods). Error bars denote SEM; *n* = 3.

of supplied mevalonic acid, likely owing to efficient biosynthesis of mevalonic acid (21 mM in broth) (Materials and Methods).

Product Addition Prevents Production Declines on a Long-Term Scale. To test the performance of our mevalonic acid addition system, we experimentally simulated durations of scale-up from the master cell bank to large production bioreactors (>200 m³ volume) by serial passaging (8) and compared the addicted *e3.9* strain to the nonaddicted control strain *pe1* with wild-type *folP-glmM* promoter. To avoid stationary-phase cultures not commonly desired in industrial processes, we passaged the four parallel lineages of each strain in exponential phase strictly every 16 h for a total of 14 times, while freeze-storing samples for subsequent analysis (Materials and Methods). A 2% volume was transferred at each passage (0.3×10^8 – 0.9×10^8 cells) (Materials and Methods) to minimize the diversity-limiting effects of passaging bottlenecks and hence also the potential bias in evolutionary dynamics. At the beginning of the experimental simulation, *pe1* and *e3.9* displayed equal growth rates, dividing on average 5.5 times (generations) per passage (SI Appendix, Table S5) in agreement with design criterion 3; however, toward the final passages, *pe1* lineages accumulated slightly more biomass per passage (SI Appendix, Table S5), indicating a gain of fitness that was absent for the product-addicted strain *e3.9*. To investigate these dynamics, we quantified the population maximum growth rates over the long-term experiment (Materials and Methods). All *pe1* lineages gradually increased maximum growth rates until they reached a new stable plateau of nearly double maximum growth rates around generation 80 (Fig. 3A). This new fitness state could result from a production load alleviated by

subpopulations of detrimental genetic variants as previously described in long-term cultivations (8).

Next, we recultivated the stocked cell samples to investigate the mevalonic acid production dynamics over the long-term experiment. As designed for criterion 2, initial production in product-addicted and nonaddicted strains was equal at, respectively, 2.9 and 3.1 g/L mevalonic acid (Materials and Methods and Fig. 3B and D), corresponding to a yield of 0.38 g/g glucose (70% of the theoretical maximum). However, in accordance with their rising growth rates during the long-term experiment (Fig. 3A), the *pe1* lineages gradually lost mevalonic acid production (Fig. 3B), likely due to selective enrichment of nonproducing cells in the populations (8). Overall, these production declines corresponded to a half-life of the population productivity of 50 generations. In all four nonaddicted lineages, mevalonic acid production ultimately fell to below 5% within the accumulated 95 generations studied. In contrast, the four product-addicted lineages of *e3.9* retained their initial low growth rates and effectively endured 95 generations without statistically significant improvements of maximum growth rates (Fig. 3C). Coherent with not gaining fitness, the four product-addicted lineages remained >95% productive at the end of the large-scale simulated fermentation at generation 95 (Fig. 3D). Thus, these addicted populations maintained the functional metabolic pathway for a significantly prolonged cultivation period, postponing the beginning of a decline from 50 to at

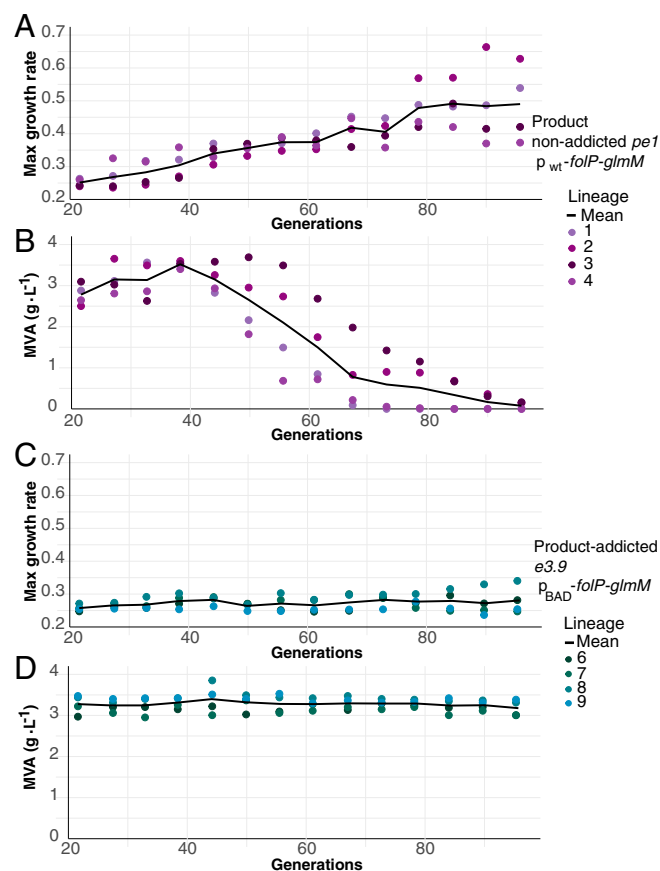


Fig. 3. Product addition prevents evolutionary fitness gains that lead to mevalonic acid (MVA) production decline at scale, as seen in experimentally simulated long-term fermentations. (A) Maximum growth rate (h⁻¹) for a long-term cultivated mevalonic-acid-producing, nonaddicted *pe1* strain (mean and individual four lineages shown). (B) Mevalonic acid production of *pe1* (mean and individual four lineages shown). (C) Maximum growth rate (h⁻¹) for long-term cultivated mevalonic-acid-producing, addicted *e3.9* strain (mean and individual four lineages shown). (D) Mevalonic acid production of *e3.9* (mean and individual four lineages shown).

least 85 generations (Fig. 3 *B* and *D*). This gain corresponds to a massive increase in terms of functional working volume due to exponential growth: 60 generations of cultivation would match the cell divisions required to saturate a 200-m³ bioreactor at high cell density. This evasion of otherwise evolving production declines strongly indicates that the product addition design functioned in the manner intended, synthetically confining the cells to the otherwise unfavorable state of high-yield metabolite production.

Product Addition Limits Accumulation of Genetic Pathway Heterogeneities. Genetic heterogeneities in populations of engineered bioproduction strains can be uncovered by matching mathematical models with deep sequencing and algorithms for population-level assessment of structural genetic variation and SNPs (8). We wanted to explore whether the product addition system suppressed accumulation of such genetic heterogeneities in the population. Accordingly, we deep-sequenced (>1,000-fold average coverage, *SI Appendix*, Table S6) the pathway populations at three time points during the long-term experiment using short-read (Illumina) DNA sequencing. We found that mobile elements in the populations of the nonaddicted production lineages enriched over the course of the simulated fermentations. They reached 94–100% presence in the production plasmid populations at the final sampling time (Fig. 4*A*), explaining well the simultaneous phenotypic observations of production decline (Fig. 3*A* and *B*). The plasmid disruptions were mediated by several different mobile element subtypes from the host XL1 genome, yet were mainly driven by insertion sequences (ISs) IS1 and IS10, which is in agreement with our previous observations for bacterial mevalonic acid production, by a differently encoded production pathway (8). In contrast, the pathway populations of the product-addicted cells remained genetically intact, and mobile elements were only observed at frequencies below 2% at the experimental end point (Fig. 4*A*). These genetic observations support the hypothesis that the product addition system provides a sufficient synthetic selection pressure to select against accumulation of the otherwise detrimental mobile element insertions that abolish biosynthetic capacity in full agreement with the observed phenotypic dynamics (Fig. 3).

All 14 major position-resolved disruptions (Fig. 4) were mediated by IS1 (0.2–56%) and IS10 (36–93%), while 10 additional mobile element subtypes had transposed from the host chromosome into the plasmid populations at generation 86, however, at frequencies below 0.1% (*SI Appendix*, Fig. S2).

In the populations of the product-addicted lineages, we detected only four low-frequency disruptions (0.1–1.2%) at the sequenced end point (Fig. 4*B*). One of these disruptions (IS1 in *atoB*) was already present in equal frequencies at generation 46, indicating that this allele can reside stably in the populations, perhaps since *atoB* also exists in a complementary native chromosomal copy and IS1 is known to transcriptionally activate downstream genes (8, 31). These low-frequency disruptions do not appear to affect population-level fitness and mevalonic acid production (Fig. 3 *C* and *D*); however, such deep genetic dynamics could indicate the onset of future escapes.

We also analyzed the sensor plasmid populations by DNA sequencing at high depth, as sensor mutations or disruptions might render the essential gene operon constitutive since AraC acts as both a repressor and an activator (32); however, in agreement with the observed production stability, we did not detect genetic heterogeneities in the sensor populations that would explain an escape from engineered addition.

Discussion

Synthetic product addition is a concept for linking costly, high-yield metabolite production to cellular growth by a product-responsive biosensor regulating nonconditionally essential genes. We have engineered and shown that such a design can prevent formation of detrimental genetic heterogeneity and be implemented without use of classical selection phenotypes (nutrient prototrophies or antibiotic resistance). Previous biosensor-based designs for

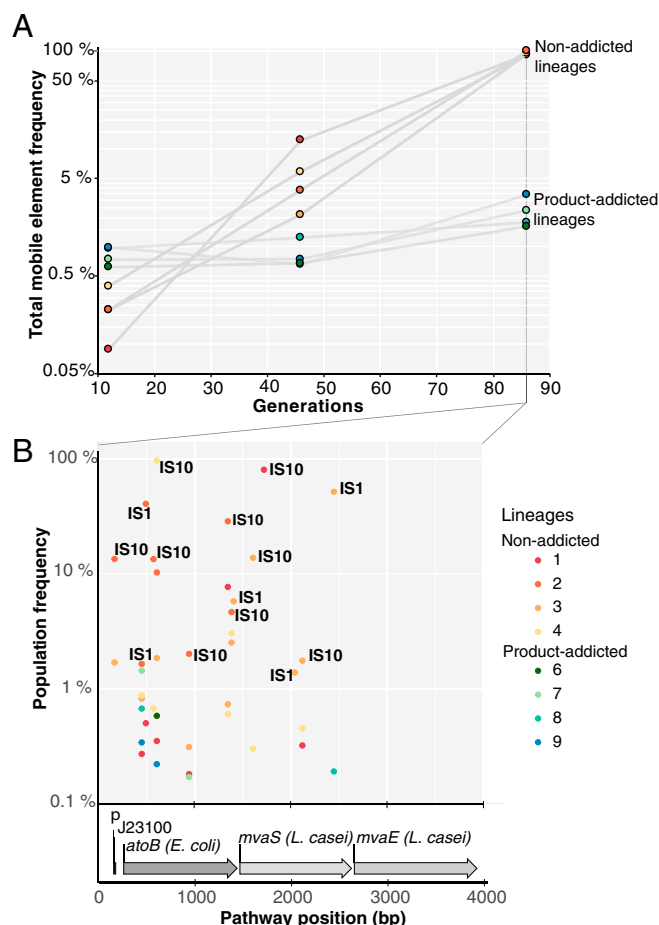


Fig. 4. Deep DNA sequencing of production pathway population reveals evaded mobile element disruptions of the pathway in product-addicted lineages over time. (*A*) Cumulative frequency of host mobile elements in plasmid populations during the simulated long-term fermentation with product-addicted and nonaddicted strains (four individual lineages shown). (*B*) Position-specific frequencies of mobile element insertions in the deep-sequenced production pathway for mevalonic acid at generation 86 (the four individual lineages shown). The identities of ISs are labeled for the highest-frequency insertion in all sites.

product monitoring have harnessed classical conditional selection genes in library-wide selections (18, 19) and for enrichment of phenotypic overproducing subpopulations (5); however, medium amendments such as antibiotics are not feasible for most fermentation products due to cost and regulatory restrictions (33). Instead, using the endogenous selective pressure of carefully tuned essential genes, we have shown that an addition design can significantly stabilize mevalonic acid production to industrially relevant cultivation scales, despite a considerable production load of a high-yielding pathway.

Although difficult to detect at a population level and on a laboratory scale, evolution is a constraining factor in the performance of bio-production strains (8, 12). Biosensor-based product addition is agnostic to the mechanisms of production declines: By assessing the product, the concept works at the level of the intended phenotypic outcome and thus redirects evolutionary forces for the benefit of an engineered strain design. Addressing such production declines mechanistically is normally a research-intensive step in bioprocess development that faces two fundamental biological factors, namely the spontaneous escape rate from the engineered pathway (e.g., by mobile element transposition, recombination, and replication errors) and the selective production load (e.g., by metabolic toxicities and burdens

Table 1. Plasmids used to generate strains

Plasmid	Genetic features	Source
pMVA1	<i>P</i> _{J23100} : <i>atoB-mvaS-mvaE:t</i> , cam ^R , p15A	8
pBAD18	araC, amp ^R	25
pBAD18-Cm	araC, cam ^R	25
pBAM18spec	araCmev, spec ^R	This study
pKD46	l-Arabinose-inducible lambda recombineering, ori ^{ts} , amp ^R	26

of biosynthesis). A genome-reduced *E. coli* strain, MDS42, free of mobile elements may, e.g., provide significant life-time extension to dispensable heterologous pathways (34), although not necessarily better starting-point production (8). Classic selection schemes with biosensors (PopQC) can enrich phenotypically better subpopulations (5), although this requires medium amendments while the necessary tuning and potential to ensure favorable evolutionary stability of such systems are unknown. Pathway toxicities may be addressed using adaptive laboratory evolution strategies or dynamic pathway activation (1, 35). Combined, these approaches require significant strain redesign and insight into the specific error modes, which makes systems for continuously maintaining correct fermentation populations appealing. Dynamic, nonconditional addiction to production is therefore an attractive alternative strategy to avoid unpredictable, detrimental error modes from limiting bioprocesses at scale.

Synthetic population maintenance should fundamentally require milder growth penalties than library-wide genetic screens, which prompted our use of endogenous cell processes as growth regulators, which are nonconditional in contrast to classical selection genes (design criterion 1). We successfully used the *folP-glmM* operon; however, other nonconditionally essential processes such as polymerases, gyrases, or toxin/antitoxin systems might also be useful. In designs for biocontainment of genetically engineered organisms, cells have been addicted to supplemented molecules, thereby preventing unintended environmental release (36–39). Such systems have utilized conditional expression of essential genes in circuits that rendered cells strictly viable only under certain conditions such as enzyme redesign for synthetic dependency on a nonconventional amino acid (37). Using endogenous cell processes, we have demonstrated that synthetic biology can be used to confine a cell to an engineered, costly production genotype. To not unduly compromise bioprocess economics, we evaluated the biosynthetic consequence of our essential process perturbation to ensure that the initial isogenic population did not lose production performance by pleiotropic effects (design criterion 2).

An important consideration for synthetic selection designs are the evolutionary forces that promote mutation of critical control nodes. This risk has previously pointed to a need for redundantly layered or toggled selections to prevent escapes due to a single mutation (18, 19, 36). In product addiction, we aimed for such redundancy by the coupling of two systems (production and its addiction), which must mutate individually before escape, while only the production system confers significant reduction in fitness (design criterion 3). Growth restrictions would also delay biomass formation and hence lower the overall process productivity, which effectively increases the required bioreactor capacity (9). A weakness of our system is the concurrent regulation of two essential genes in an operon, which means that a single mutation in the sensor promoter could cause escape despite two actuating genes. However, we did not observe this in the present study, likely due to our tuning of the system to minimize fitness cost.

Product addiction is best suited as a sentinel of product pathways that are active during phases of cellular growth and therefore costly to maintain. This relation is also important since addiction depends on sufficient sensor saturation. Pathways strictly active in stationary growth phase may inherently experience lower genetic

instability and be incompatible with a product addiction scheme. Implementation of product addiction systems requires a product-responsive biosensor with a sensitivity that dynamically matches the operational intracellular concentrations in the ideally biosynthetic cells. Sensitivity becomes especially important as extracellular product theoretically might diffuse to cross-feed nonproducing cheater cells and consequently bypass the addiction system. We therefore also searched for nonproductive subpopulations by time-lapse deep sequencing and phenotypic characterization of the fermentation lineages (Figs. 3 and 4) yet did not observe evolving escapes in the addicted lineages. Industrial practice of passing cultures to seed trains of increasing volumes before final scale likely dilutes the product at early stages (9). However, to bridge the sensitivity gap, sensor tuning may also become important to selectively match the high intracellular concentrations of commercialized cell factory strains, e.g., by directed sensor evolution (40) or signal-processing buffers (41). Furthermore, sensor mutagenesis by rational and random approaches have lately shown success in changing transcription factor specificity for recognition of new biological ligands (42, 43). This will be important to enable more matching pairs of sensors with high-yielding, loaded metabolic pathways and bring molecular biosensors to work in large-scale fermentations. Not only sensing for the pathway end product, recent molecular or whole-cell biosensors may indeed prove their worth in bioprocessing by, e.g., monitoring of lactate, glycolytic flux or pH levels, and accordingly expand the applicability of product addiction strategies (44, 45). In conclusion, we have demonstrated the concept of a synthetic product addiction system, which prevents genetic heterogeneities of nonproducing subpopulations from forming in long-term fermentation with a high-yielding mevalonic acid pathway. We anticipate that utilization of such product addiction systems could significantly aid the robust industrial scale-up of bio-production pathways to maintain a wide range of chemicals and cellular states.

Materials and Methods

Plasmids. Plasmids listed in Table 1 were used to generate *E. coli* strains, as listed in Table 2. pBAM18spec was assembled by uracil excision cloning (*SI Appendix, SI Text 2*).

Strains and Their Construction. *E. coli* XL1 (“XL1 Blue,” Agilent) was used as host: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F'* *proAB lacI*^q Δ M15 *Tn10* (*Tet*^r)].

Standard chemical transformation or electroporation was used to introduce plasmids to the strains listed in Table 2, while linear DNA was introduced as described below.

Media. Standard M9 medium was M9 medium with 0.8% glucose and 0.4% casamino acids. All media recipes given in *SI Appendix, SI Text 3*.

Cloning of Integration DNA for Promoter Replacement. Linear DNA for replacement of the essential gene promoter including four RBS variants was generated by uracil-excision cloning of PCR fragments (*SI Appendix, SI Texts 1 and 2*).

Table 2. Strains generated and analyzed in this study from an *E. coli* XL1 parent

Strain	Plasmid	Encoded sensor	<i>folP-glmM</i> promoter and RBS
<i>araC</i>	pBAD18-Cm	AraC	WT
<i>e3.5</i>	pBAD18	AraC	pBAD-RBS1
<i>e3.6</i>	pBAD18	AraC	pBAD-RBS2
<i>e3.5m</i>	pBAD18	AraC	pBAD*-RBS1
<i>e3.9</i>	pMVA1, pBAM18spec	AraCmev	pBAD*-RBS1
<i>pe1</i>	pMVA1, pBAM18spec	AraCmev	WT

RBS sequences are specified in *SI Appendix, Table S4*.

Chromosomal Engineering for Biosensor-Dependent Expression of Essential Genes. Cells were engineered for biosensor-based metabolite-dependent growth by recombineering assisted with pKD46 by transformation with linear PCR-generated integration DNA encompassing the variants of responsive *P_{BAD}* and homologous targeting flanks (*SI Appendix, SI Text 3*).

Measurement of Growth Response to L-Arabinose. All strains were streaked on LB agar supplemented with 0.2% arabinose to allow growth of addicted strains, from which single colonies were grown to exponential phase and inoculated in microtiter plate growth assay (*SI Appendix, SI Text 3*).

Continuous Growth of Mevalonic-Acid-Producing Strains. Four parallel lineages of *pe1* and *e3.9* were inoculated from single colonies into M9 medium, which was cultured at 30 °C with horizontal shaking (250 rpm) in a Labnet 311DS incubator. For initial synchronization, individual first cultures were taken out of the incubator for a few hours to reach approximately the same OD₆₀₀ at the time of passaging. Each culture was 25 mL in volume and was grown for 16 h at 30 °C with horizontal shaking (250 rpm) in a Labnet 311DS incubator. After 16 h, 0.5 mL of broth was passed into 25 mL of fresh medium and incubated under the same conditions for another 16 h. An OD₆₀₀ reading on the Synergy H1 plate reader was taken to monitor growth progression (*SI Appendix, Table S5*) and ensure that the culture was still in exponential growth phase and avoid a subsequent lag phase. Upon each passage to fresh medium, a glycerol stock of each culture was stored at –80 °C. Broth culture (1.8 mL) was freeze-stored for subsequent deep DNA sequencing.

Deep DNA Sequencing and Analysis. Production plasmid populations were purified from chosen time points. The plasmid populations were prepared for Miseq sequencing using the Nextera XT v2 set A kit (Illumina) according to the manufacturer's instructions with the addition of two extra "limited-cycle PCR" cycles. Sequencing was performed in a pooled run with a 150-bp paired-end reading (*SI Appendix, SI Text 3*).

Measurement of Mevalonic Acid Production and Population Growth Rate. Following the simulated fermentation, each population sample from a 25-μL glycerol stock was used to inoculate 15 mL of M9 medium, and the culture was incubated at 30 °C with shaking at 250 rpm in a Labnet 311DS incubator for 100 h. Subsequently, mevalonic acid was quantified on an Ultimate 3000 HPLC running a 5-mM sulfuric acid mobile phase on an Aminex HPLC-87H ion exclusion column (*SI Appendix, SI Text 3*). Maximum population growth rates were measured in a microtiter plate reader inoculated from the production cultures (*SI Appendix, SI Text 3*).

ACKNOWLEDGMENTS. We thank Anna Koza for operation of the Miseq DNA-sequencing instrument and Mette Kristensen for help with HPLC analysis. The research leading to these results has received funding from the Novo Nordisk Foundation, Denmark, and from the European Union Seventh Framework Programme (FP7-KBBE-2013-7-single-stage) under Grant 613745, Promys.

- Lee SY, Kim HU (2015) Systems strategies for developing industrial microbial strains. *Nat Biotechnol* 33:1061–1072.
- Nielsen J, Keasling JD (2016) Engineering cellular metabolism. *Cell* 164:1185–1197.
- Heins A-L, Lencastre Fernandes R, Gernaey KV, Lantz AE (2015) Experimental and in silico investigation of population heterogeneity in continuous *Saccharomyces cerevisiae* scale-down fermentation in a two-compartment setup. *J Chem Technol Biotechnol* 90:324–340.
- Mustafi N, Grünberger A, Kohlheyer D, Bott M, Frunzke J (2012) The development and application of a single-cell biosensor for the detection of L-methionine and branched-chain amino acids. *Metab Eng* 14:449–457.
- Xiao Y, Bowen CH, Liu D, Zhang F (2016) Exploiting nongenetic cell-to-cell variation for enhanced biosynthesis. *Nat Chem Biol* 12:339–344.
- Glick BR (1995) Metabolic load and heterologous gene expression. *Biotechnol Adv* 13:247–261.
- Pitera DJ, Paddon CJ, Newman JD, Keasling JD (2007) Balancing a heterologous mevalonate pathway for improved isoprenoid production in *Escherichia coli*. *Metab Eng* 9:193–207.
- Rugbjerg P, Myling-Petersen N, Porse A, Sarup-Lytzen K, Sommer M (2018) Diverse genetic error modes constrain large-scale bio-based production. *Nat Commun*, 10.1038/s41467-018-03232-w.
- Ikeda M (2003) Amino acid production processes. *Adv Biochem Eng Biotechnol* 79:1–35.
- Bull JJ, Barrick JE (2017) Arresting evolution. *Trends Genet* 33:910–920.
- Azuma T, Nakanishi T, Sugimoto M (1988) Isolation and characterization of a stable L-arginine producer from continuous culture broth of *Corynebacterium acetoacidophilum*. *J Ferment Technol* 66:279–284.
- Zelder O, Hauer B (2000) Environmentally directed mutations and their impact on industrial biotransformation and fermentation processes. *Curr Opin Microbiol* 3:248–251.
- Farmer WR, Liao JC (2000) Improving lycopene production in *Escherichia coli* by engineering metabolic control. *Nat Biotechnol* 18:533–537.
- Dahl RH, et al. (2013) Engineering dynamic pathway regulation using stress-response promoters. *Nat Biotechnol* 31:1039–1046.
- Lo T-M, Chng SH, Teo WS, Cho H-S, Chang MW (2016) A two-layer gene circuit for decoupling cell growth from metabolite production. *Cell Syst* 3:133–143.
- Conrad TM, Lewis NE, Palsson BO (2011) Microbial laboratory evolution in the era of genome-scale science. *Mol Syst Biol* 7:509.
- Michener JK, Thodey K, Liang JC, Smolke CD (2012) Applications of genetically-encoded biosensors for the construction and control of biosynthetic pathways. *Metab Eng* 14:212–222.
- Raman S, Rogers JK, Taylor ND, Church GM (2014) Evolution-guided optimization of biosynthetic pathways. *Proc Natl Acad Sci USA* 111:17803–17808.
- Genee HJ, et al. (2016) Functional mining of transporters using synthetic selections. *Nat Chem Biol* 12:1015–1022.
- Rogers JK, Church GM (2016) Multiplexed engineering in biology. *Trends Biotechnol* 34:198–206.
- Ceroni F, Algar R, Stan G-B, Ellis T (2015) Quantifying cellular capacity identifies gene expression designs with reduced burden. *Nat Methods* 12:415–418.
- Borkowski O, Ceroni F, Stan GB, Ellis T (2016) Overloaded and stressed: Whole-cell considerations for bacterial synthetic biology. *Curr Opin Microbiol* 33:123–130.
- Mengin-Lecreux D, van Heijenoort J (1996) Characterization of the essential gene *glmM* encoding phosphoglucosamine mutase in *Escherichia coli*. *J Biol Chem* 271:32–39.
- Dallas WS, Gowen JE, Ray PH, Cox MJ, Dev IK (1992) Cloning, sequencing, and enhanced expression of the dihydropteroate synthase gene of *Escherichia coli* MC4100. *J Bacteriol* 174:5961–5970.
- Guzman LM, Belin D, Carson MJ, Beckwith J (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose *P_{BAD}* promoter. *J Bacteriol* 177:4121–4130.
- Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 97:6640–6645.
- Bonde MT, et al. (2016) Predictable tuning of protein expression in bacteria. *Nat Methods* 13:233–236.
- Tang S-Y, Cirino PC (2011) Design and application of a mevalonate-responsive regulatory protein. *Angew Chem Int Ed Engl* 50:1084–1086.
- Martin VJJ, Pitera DJ, Withers ST, Newman JD, Keasling JD (2003) Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. *Nat Biotechnol* 21:796–802.
- Xiong M, Schneiderman DK, Bates FS, Hillmyer MA, Zhang K (2014) Scalable production of mechanically tunable block polymers from sugar. *Proc Natl Acad Sci USA* 111:8357–8362.
- Mahillon J, Chandler M (1998) Insertion sequences. *Microbiol Mol Biol Rev* 62:725–774.
- Ogden S, Haggerty D, Stoner CM, Kolodrubetz D, Schleif R (1980) The *Escherichia coli* L-arabinose operon: Binding sites of the regulatory proteins and a mechanism of positive and negative regulation. *Proc Natl Acad Sci USA* 77:3346–3350.
- Rosano GL, Ceccarelli EA (2014) Recombinant protein expression in *Escherichia coli*: Advances and challenges. *Front Microbiol* 5:172.
- Pósfai G, et al. (2006) Emergent properties of reduced-genome *Escherichia coli*. *Science* 312:1044–1046.
- Portnoy VA, Bezzan D, Zengler K (2011) Adaptive laboratory evolution: Harnessing the power of biology for metabolic engineering. *Curr Opin Biotechnol* 22:590–594.
- Gallagher RR, Patel JR, Interiano AL, Rovner AJ, Isaacs FJ (2015) Multilayered genetic safeguards limit growth of microorganisms to defined environments. *Nucleic Acids Res* 43:1945–1954.
- Mandell DJ, et al. (2015) Biocontainment of genetically modified organisms by synthetic protein design. *Nature* 518:55–60.
- Agmon N, et al. (2017) Low escape-rate genome safeguards with minimal molecular perturbation of *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* 114:E1470–E1479.
- Molin S, et al. (1993) Suicidal genetic elements and their use in biological containment of bacteria. *Annu Rev Microbiol* 47:139–166.
- Collins CH, Arnold FH, Leadbetter JR (2005) Directed evolution of *Vibrio fischeri* LuxR for increased sensitivity to a broad spectrum of acyl-homoserine lactones. *Mol Microbiol* 55:712–723.
- Rugbjerg P, Genee HJ, Jensen K, Sarup-Lytzen K, Sommer MOA (2016) Molecular buffers permit sensitivity tuning and inversion of riboswitch signals. *ACS Synth Biol* 5:632–638.
- Taylor ND, et al. (2016) Engineering an allosteric transcription factor to respond to new ligands. *Nat Methods* 13:177–183.
- Feng J, et al. (2015) A general strategy to construct small molecule biosensors in eukaryotes. *eLife* 4:7250–7257.
- Lehning CE, Siedler S, Ellabaan MMH, Sommer MOA (2017) Assessing glycolytic flux alterations resulting from genetic perturbations in *E. coli* using a biosensor. *Metab Eng* 42:194–202.
- Dekker L, Polizzi KM (2017) Sense and sensitivity in bioprocessing-detecting cellular metabolites with biosensors. *Curr Opin Chem Biol* 40:31–36.